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(54) Title: NOVEL INSULIN DERIVATIVES

(57) Abstract

Exchanging asparagine in the A21 position of insulin into another amino acid gives novel insulin derivatives which are more stable and less immunogenic than the parent compound. The insulin derivatives can be prepared by transpeptidation of a biosynthetic precursor which may be expressed in a host organism such as a yeast.

# NOVEL INSULIN DERIVATIVES

## TECHNICAL FIELD

The present invention relates to novel insulin derivatives having improved properties, to methods for their preparation and to preparations containing such novel insulin derivatives.

## BACKGROUND OF THE INVENTION

In the treatment of diabetes mellitus, many varieties of insulin preparations have been suggested and used. Even though improved insulin preparations have steadily been invented during the insulin era, there is still a need for insulin preparations with improved properties.

Acidic solutions of insulin have been used earlier, both as short-acting preparations and together with protamine and/or zinc as long-acting preparations. However, under ordinary circumstances the chemical stability of insulin at pH values below 4.5 is low, as formation of desamidoinsulins (Sundby, F., J.Biol.Chem. 237 (1962), 3406 - 3411) and covalent dimers (Steiner et al., Diabetes 17 (1968), 725 - 736) takes place. In the pH range 4.5 - 6.5, insulin precipitates. Hence, in order to obtain soluble short-acting insulin preparations (by the addition of blood-flow enhancing agents) and long-acting insulin preparations (by the addition of protamine and/or zinc, an insulin stable at a low pH would be desirable.

One object of this invention is to provide insulin derivatives with improved properties.

A second object of this invention is to provide solutions of insulin derivatives having an improved stability.

A third object of this invention is to provide preparations of insulin derivatives with low or with no immunogenic activity.

A fourth object of this invention is to provide insulin preparations which are soluble at pH values from about 2.0 to about 8.0, preferably from about 2.0 to about 4.5 and from about 6.5 to about 8.0.

A fifth object of this invention is to provide solutions of insulin derivatives having an improved stability at pH values of about 3-4.

A sixth object of this invention is to provide long-acting solutions of insulin derivatives.

## STATEMENT OF THIS INVENTION

The present invention relates to human, porcine, rabbit and des(B30) insulin wherein the A21 amino acid has been substituted by Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr, Val or hSer.

Such compounds can be designated by the general formula I

INSUL-A21
:
B30 (I)

wherein INSUL represents des(A21),des(B30) human insulin and A21 represents one of the amino acids Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr, Val or hSer connected to $Cys^{A20}$ in INSUL, and B30 represents hydrogen or one of the amino acids Ser, Ala or Thr connected to $Lys^{B29}$ in INSUL.

It is known that during the acidic ethanol extraction of mammalian insulins many dimers are formed (Steiner) and, furthermore, monodesamidoinsulins are formed under acid conditions (Sundby).

It has now, surprisingly, been found that the formation of such undesired dimers is substantially reduced or almost eliminated when the insulin compound used is one of the above insulin derivatives wherein $Asn^{A21}$ has been exchanged with one of the above-mentioned amino acids. This substitution also eliminates the formation of monodesamido insulins.

The novel insulin derivatives have the following advantages:

1) The formation of the immunogenic dimers, i.e. covalently linked insulin molecules linked either through the two A-chains, (AA) dimer, or through one A-chain and one B-chain, (AB) dimer, (Helbig, H.J., Deutsche Wollforschungsinstitut, dissertation, 1976) is substantially eliminated (a chromatographic fraction of crude porcine insulin, the b-component, containing the dimers was shown to be immunogenic in rabbits (Schlichtkrull et al., Horm.Metab.Res. Suppl. 5 (1974), 134 - 143)).

2) The stability of the novel insulin derivatives is so high that it will probably be possible to store preparations containing these novel insulin derivatives at room temperature for a long period of time. This will be a major advantage for the patient.

3) It will be possible to prepare dissolved preparations containing the novel insulin derivatives at pH values from about 2 to about 8, preferably in the range from about 2 to about 4.5 and above 6.5.

4) It will be possible to prepare preparations containing the novel insulin derivatives which, at pH values of about 3, have a substantially improved chemical stability.

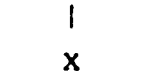
5) In the pH range of about 3-4, which is inappropriate for mammalian insulin because of chemical instability, useful solutions of insulin derivatives can be made in the presence of magnesium ions in concentrations of about 0.005 M to 0.5 M.

6) It will be possible to prepare soluble, rapidly acting preparations containing the novel insulin derivatives by the addition of compounds which enhance the absorption.

7) It will be possible to prepare soluble, retarded preparations containing the novel insulin derivatives by the addition of zinc and/or protamine to acid solutions, i.e. solutions having a pH value in the range from about 2.5 to about 4.

8) It will be possible to prepare preparations containing the novel insulin derivatives having different profiles.

Compounds of formula I may be prepared by a transpeptidation reaction in which a biosynthetic precursor compound having the general formula II



(II)

wherein A21 is as defined above, and X is a bond, an amino acid residue or a peptide residue bridging the carboxyl group of $Lys^{B29}$ to the amino group of $Gly^{A1}$, is reacted with an amino compound of the general formula III

(III)

wherein Z is Thr, Ala or Ser wherein any hydroxy group may be protected, and R is a carboxy protecting group (e.g. methyl or tert-butyl), using trypsin or a trypsin-like enzyme as a catalyst in a mixture of water and organic solvents analogously as described in US patent specification No. 4,343,898, whereafter the carboxy protecting group and any hydroxy protecting group is removed. X may for example be a moiety of the formula IV

$$-(Q_q-K)_r \qquad (IV)$$

wherein Q is a peptide chain with q amino acids, q is an integer from 0 to 33, K is Lys or Arg, and r is zero or one.

Compounds of formula II may be prepared by a method similar to the method described in European patent application Nos. 163,529 and 214.826. By this method a DNA-sequence encoding a compound with the formula II is inserted into a suitable expression vector which, when transferred to a suitable yeast strain, is capable of expressing the desired compound with correctly positioned disulphide bridges. The product expressed is then isolated from the cells or the culture broth depending on whether it is secreted from the cells or not.

At neutral pH, compounds of formula I have the same charge as human insulin. In solution, compounds of formula I may be present as hexamers.

Examples of specific preferred compounds according to this invention are the following: $Gly^{A21}$ human insulin, $Ala^{A21}$ human insulin, $Ser^{A21}$ human insulin, $Thr^{A21}$ human insulin, $hSer^{A21}$ human insulin, $Gly^{A21}$ porcine insulin, $Ala^{A21}$ porcine insulin, $Ser^{A21}$ porcine insulin and $Thr^{A21}$ porcine insulin.

Insulin preparations of this invention can be prepared by dissolving a compound of formula I in an aqueous medium at slightly acidic conditions, for example, in a concentration of from about 240 to about 600 nmole/ml.

The aqueous medium can be made isotonic by the addition of sodium chloride, sodium acetate or glycerol.

If a protracted preparation is required the above mentioned isotonic agents can in part or completely be replaced by a zinc salt or a mixture of zinc salts at a concentration of up to about 5 µg $Zn^{2+}$ per nmol of compound of formula I.

Further, it has been found that many magnesium salts have a solubilising effect on insulin at pH values of from about 4 to about 6.2 and an enhancing effect on the absorption of insulin. Various mixtures of magnesium salts have the same effect. It is, therefore, concluded that the presence of magnesium ions at certain concentrations is a critical parameter for the solubility of insulin at pH values of from about 4 to about 6.2 and for the rate of absorption. The range of applicable magnesium ion concentration is from about 0.005 M to about 0.5 M, preferably above 0.05 M. The upper limit is somewhat arbitrary being chosen from the assumption that in some cases (e.g. for intraperitoneal infusion) some overstepping of isotonicity may be acceptable. According to a preferred embodiment of this invention the preparations contain magnesium ions in a concentration of from about 0.08 M to about 0.3 M.

It has furthermore been found that protracted - or further protracted - preparations of the insulin derivatives of this invention are obtained when protamine is added to the above mentioned preparations, i.e. the preparations containing no zinc ions and no magnesium ions, the preparations containing zinc ions and the preparations containing magnesium ions. The amount of protamine to be used is from about 5% to about 50%, preferably from about 8% to about 40%, more preferred from about 10% to about 30% on the basis of insulin (weight/weight).

Insulin preparations with enhanced absorption properties can also be obtained by the addition of arginine or lysine to an aqueous solution of the insulin. The preferred concentration of these amino acids is from about 0.01 M to about 0.2 M.

The insulin preparations may further contain buffers such as acetate and citrate and preservatives such as phenol, m-cresol and methyl paraben. The pH of the solution is adjusted to the desired value and the insulin preparation is made sterile by sterile filtration.

Insulin solutions of this invention having a pH value in the range 3 - 6.2 may also be particularly useful for the purpose of infusion by means of pumps, because of a lack of insulin precipitation caused by carbon dioxide diffusion through catheters. Such precipitation has been observed occasionally with neutral infusion solutions, and is believed to be attributable to the lowering of the pH value caused by carbon dioxide.

The abbreviations used herein for the amino acid residues are those stated in J.Biol.Chem. 243 (1968), 3558. The amino acids stated herein are in L configuration. Within the context of this invention the term insulin when used in a plural or generic sense is intended to encompass both naturally occuring insulins and insulin derivatives. $Gly^{A21}$ human insulin is human insulin wherein $Asn^{A21}$ has been exchanged by Gly and similarly for similar names.

The insulin preparations of this invention can be used in the treatment of diabetes. It is recommended that the dosage of the insulin preparations of this invention be selected by a physician similarly to the selection of the dosage of known insulin preparations for injection.

Any novel feature or combination of features described herein is considered essential to this invention.

## Example 1
## Preparation of $Gly^{A21}$ Human Insulin

$Gly^{A21}$ human insulin was prepared by transpeptidation of a compound which according to formula II can be formulated as

$$\text{INSUL-Gly}^{A21}$$
$$\text{|} \qquad \text{(V)}$$
$$\text{Ala-Ala-Lys-}$$

wherein the terminal Ala of the bridging petide is linked to the carboxyl group of $Lys^{B29}$ and Lys is linked to the amino group of $Gly^{A1}$, with Thr-OMe (L-threonine methylester) followed by hydrolysis of the ester group with aqueous sodium hydroxide. Thus 100 mg of the compound of formula V was dissolved in 0.5 ml of 10 M acetic acid and 1 ml of 2 M Thr-OMe in N,N-dimethylacetamide was added. The mixture was cooled to 12°C. 10 mg of trypsin dissolved in 0.2 ml of 0.05 M calcium acetate was added. After 48 hours at 12°C the proteins were precipitated by addition of 20 ml of acetone. The conversion of the starting material into $Gly^{A21}$-$(Thr\text{-}OMe)^{B30}$ human insulin was 88% by HPLC.

250 mg of $Gly^{A21}$-$(Thr\text{-}OMe)^{B30}$ human insulin was suspended in 25 ml of water and dissolved by the addition of 1 N sodium hydroxide solution to a pH value of 10.0. The pH value is kept constant at 10.0 for 24 hours at 25°C. The insulin derivative formed was crystallized by the addition of 2 g of sodium chloride, 350 mg of sodium acetate trihydrate and 2.5 mg of zinc acetate dihydrate followed by the addition of 1 N hydrochloric acid to obtain a pH value of 5.52. After 24 hours at 4°C the crystallized material was isolated by centrifugation washed with 3 ml of water, isolated by centrifugation, and dried in vacuo. Yield: 210 mg of $Gly^{A21}$ human insulin.

The compound of formula V was prepared by a method analogous to example 2 of European patent application No. 214.826.

Example 2

Preparation of Injectable Solution of Compounds of Formula I

15 μmol of $Gly^{A21}$ human insulin containing 0.5% of zinc are dissolved in water (5 ml) containing hydrochloric acid (80 μl of 1 N) followed by the addition of an aqueous solution (10 ml) containing phenol (65 mg) and glycerol (400 mg). The pH value of the solution is adjusted to 3.0 by means of a sodium hydroxide solution and the total volume is adjusted to 25 ml with water. The resulting solution is sterilized by filtration and subsequently transferred aseptically to vials (5 ml).

Example 3

Soluble Preparation of $Gly^{A21}$ Human Insulin with Protracted Action

15 μmol of $Gly^{A21}$ human insulin (zinc free) are dissolved in water (5 ml). To this solution is added hydrochloric acid (80 μl of 1 N) and zinc chloride (100 μl of 0.6 M) followed by the addition of an aqueous solution (15 ml) containing protamine sulphate (37 mg), m-cresol (50 mg) and sodium chloride (200 mg). The pH is adjusted to 3.5 with sodium hydroxide solution and the total volume is adjusted to 25 ml with water. Finally, the solution is sterilized by filtration and transferred aseptically to sterile vials.

The absorption profile after subcutaneous injection in pigs was found comparable to that of the well known insulin suspension Protaphane HM 100 IU/ml.

Example 4

Soluble Preparation of $Gly^{A21}$ Human Insulin with Fast Action

15 µmol of $Gly^{A21}$ human insulin (zinc free) are dissolved in water (10 ml). To this solution is added hydrochloric acid (40 µl of 1 N) and magnesium chloride (2.6 ml of 1 M) followed by the addition of an aqueous solution of benzyl alcohol (8 ml of 0.3 M). The pH is adjusted to 5.7 with sodium hydroxide solution and the total volume is adjusted to 25 ml with water. Finally the solution is sterilized by filtration and transferred aseptically to sterile vials.

Example 5

Chemical Stability of $Gly^{A21}$ Human Insulin in Preparations

Three preparations containing 0.24 mM of $Gly^{A21}$ human insulin (zinc free), 0.26% (w/v) of phenol and 1.6 % (w/v) of glycerol were prepared and their pH value adjusted to 3.0, 4.0, and 5.0, respectively.

Samples were analyzed after storage at 45°C for two weeks using human insulin preparations of the same composition as reference.

Table 1 shows the content of insulin dimerization and polymerization products as determined by HPSEC (High Performance Size Exclusion Chromatography).

Table 2 shows the content of insulin deamidation products determined by DISC PAGE (Poly Acrylamide Gel Electrophoresis).

Table 1

| pH of Preparation | Human Insulin | $Gly^{A21}$ Human Insulin |
|---|---|---|
| 3.0 | 4.9% | 0.31% |
| 4.0 | 41.6% | 1.0% |
| 5.0 | 16.1% | 2.8% |
| Dry Insulin | 0.29% | 0.05% |

Table 2

| pH of Preparation | Human Insulin | $Gly^{A21}$ Human Insulin |
|---|---|---|
| 3.0 | 90% | 2% |
| 4.0 | 40% | 3% |
| 5.0 | 3% | 4% |
| Dry Insulin | 0.5% | 0.5% |

Example 6

Biological Potency of $Gly^{A21}$ Human Insulin

Investigation according to the British Pharmacopeia, 1980 edition, of the potency of $Gly^{A21}$ human insulin showed that this was approximately 85% of that of human insulin. Within the dose range relevant for therapeutic purposes no toxic manifestations were observed.

Example 7

Soluble Preparation of $Gly^{A21}$ Human Insulin with Further Protracted Action

15 µmol of $Gly^{A21}$ human insulin (zinc free) are dissolved in water (5 ml). To this solution is added hydrochloric acid (80 µl of 1 N) and zinc chloride (100 µl of 0.6 M) followed by the addition of an aqueous solution (15 ml) containing protamine sulphate (37 mg), m-cresol (50 mg) and magnesium chloride (200 mg). The pH is adjusted to 3.5 and the total volume is adjusted to 25 ml with water. Finally, the solution is sterilized by filtration and transferred aseptically to sterile vials.

The absorption of this preparation after subcutaneous injection in pigs was found to be substantially slower than that of the well known insulin suspension Protaphane®HM 100 IU/ml.

CLAIMS

1. Insulin derivatives of the general formula I

INSUL-A21
|
B30 (I)

wherein INSUL represents des(A21),des(B30) human insulin, characterized in that A21 represents one of the amino acids Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr, Val or hSer connected to $Cys^{A20}$ in INSUL, and B30 represents hydrogen or one of the amino acids Ser, Ala or Thr connected to $Lys^{B29}$ in INSUL, and preferably A21 is different from Phe.

2. Insulin derivatives according to Claim 1, wherein A21 represents Gly, Ala, Ser, Thr or hSer, and B30 represents Ala or Thr.

3. Preparation characterized in that it contains a compound of formula I stated in Claim 1 or 2 above with the definitions stated therein.

4. Preparation according to Claim 3, characterized in that it is soluble.

5. Preparation according to Claim 4, characterized in that it contains a compound which enhances the absorption.

6. Preparation according to claim 5, characterized in that said compound is a magnesium salt.

7. Preparation according to claim 6, characterized in that it is a solution with a pH value in the range of about 3-4 and that it contains magnesium ions Pin a concentration of about 0.005 M to about 0.5 M which preparation preferably contains a compound of formula I wherein A21 is different from Gln.

8. Preparation according to claim 5, characterized in that said compound is arginine or lysine.

9. Preparation according to Claim 3, 4, 6, 7 or 8 characterized in that it contains zinc ions and/or protamine.

10. Preparation according to any one of the claims 3-9, characterized in that it has a pH value in the range of from about 2.0 to about 8, preferably from about 2.5 to about 8.

11. Preparation according to claim 10, characterized in that it has a pH value in the range of from about 2.5 to about 4.5 or from about 6.5 to about 8.0.

12. Method for the preparation of insulin derivatives according to claim 1, wherein a biosynthetic precursor compound having the general formula II

INSUL-A21
|
X (II)

wherein A21 is as defined in claim 1, and X is a bond, an amino acid residue or a peptide residue bridging the carboxyl group of $Lys^{B29}$ to the amino group of $Gly^{A1}$, is reacted with an amino compound of the general formula III

Z-OR (III)

wherein Z is Thr, Ala or Ser wherein any hydroxy group may be protected, and R is a carboxy protecting group, using trypsin or a trypsin-like enzyme as a catalyst in a mixture of water and organic solvents whereafter the carboxy protecting group and any hydroxy protecting group is removed.

13. Method according to claim 12, wherein X is a moiety of the formula IV

$$-(Q_q-K)_r- \quad \text{(IV)}$$

wherein Q is a peptide chain with q amino acids, q is an integer from 0 to 33, K is Lys or Arg, and r is zero or one.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00033

## I CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

C07K 7/40, A61K 37/26, C12P 21/02

## II FIELDS SEARCHED

### Minimum Documentation Searched †

| Classification System | Classification Symbols |
|---|---|
| IPC 4 | A61K 37/26; C07C 103/52; C07K 7/40, 7/42; C12P 21/02; |
| US Cl | 260:112.7; 195:29; 424:178; 435:68-71; 514:3,4; 530:303-305 |

### Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ‡

SE, NO, DK, FI classes as above.

## III DOCUMENTS CONSIDERED TO BE RELEVANT §

| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages †† | Relevant to Claim No †† |
|---|---|---|
| X | GB, A, 1 453 454 (HOECHST AKTIENGESELLSCHAFT) 20 October 1976 & NL, 7314433; FR, 2204616; DE, 2252157; BE, 806522; US, 3883496; AT, 329780; CA, 1011734; CH, 602597; JP, 49080090 | 1-2, 12-13 |
| Y | DE,C2,3 104 949 (NOVO INDUSTRI A/S) 26 November 1981 & BE, 887480; FR, 2475542; SE, 8100926; SE, 427025; SE, 8100928 | 1-2, 12-13 |

* Special categories of cited documents: ††

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 1988-05-24 | 1988 -06- 01 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| Swedish Patent Office | Elisabeth Carlborg |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
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| Y | US, A, 3 364 116 (MIKLOS BODANSZKY)<br>16 January 1968 | 1-5, 9-11 |
| Y | GB,A, 2 094 145 (NOVO INDUSTRI A/S)<br>15 September 1982<br>& EP, 0060141<br>FR, 2501507<br>BE, 892413<br>JP, 57209227<br>CA, 1170990<br>US, 4472385<br>CH, 652031<br>AU, 550068 | 3-7, 9-11 |
| Y | SE,B, 378 066 (ELI LILLY AND COMPANY)<br>18 August 1975<br>& NL, 7205865<br>FR, 2134658<br>DE, 2219635<br>US, 3758683<br>GB, 1385086<br>US, 3868358<br>CH, 566784<br>CA, 976085<br>BE, 782651 | 9-11 |